

# Nor Necrosis Factor-related Apoptosis-inducing Ligand-mediated Apoptosis in Androgen-independent Prostate Cancer Cells<sup>1</sup>

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## ABSTRACT

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systemic toxicity. Thus, the challenge today is to identify or develop a cytotoxic agent that selectively induces apoptosis in metastatic prostate cancer cells but avoids significant toxicity to the normal tissues.

TRAIL (also called Apo-2L) is a type II transmembrane protein that was initially identified by searching an expressed sequence tag database with the most conserved extracellular sequence of TNF family (10, 11). Human TRAIL consists of 281 amino acids and shows highest homology with FasL (28%), followed by TNF (23%), and LT $\alpha$  (23%). The transcripts of TRAIL have been detected in many human tissues such as spleen, thymus, prostate, and lung (10, 11). To date, at least five receptors for human TRAIL have been identified, including DR4 (12), DRS (also called Apo-2, TRAIL-R2, TRICK2, or KILLER; Refs. 13 and 14), DcR1 (also called TRID, TRAIL-R3, or LIT; Refs. 15 and 16), DcR2 (also called TRAIL-R4 or TRUNDD; Ref. 17), and TR1 (18). Ligation of TRAIL to DR4 or DRS induces trimerization of the receptor, which further interacts with downstream death domain-containing adapter proteins leading to the activation of caspases (cysteine-dependent aspartate-directed proteases) and apoptotic cell death (19). In contrast to DR4 and DRS, DcR1 and DcR2 contain an incomplete cytoplasmic death domain and have been reported to prevent TRAIL-induced apoptosis, presumably by competing with DR4 and DRS for binding to TRAIL (15–17). Similar to TNF and FasL, TRAIL induces apoptosis in a wide variety of transformed or cancerous cells (10, 11). However, unlike TNF and FasL, TRAIL shows little cytotoxicity to the normal cells (10). Most interestingly, in contrast to the severe inflammatory response syndrome induced by systemically administered TNF and the fulminant hepatotoxicity of FasL, administration of TRAIL to mice seems to be devoid of systemic toxicity but shows antitumor activity (20). Thus, TRAIL may be a new promising candidate for treatment of cancer.

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**Cells, Plasmids, and Chemicals.** Human prostate cancer PC-3 and DU145 cells were obtained from American Type Culture Collection (Manassas, VA) and were cultured in MEM supplemented with 10% fetal bovine serum, 2.2 g/l sodium bicarbonate, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. Medium was normally changed to fresh medium 2 h before the treatment. Plasmids pCMV1-FLAG-DR4, pcDNA3-HA-JNK1, and pcDNA3-FLAG-JNK1(APF), rabbit anti-JNK1 antiserum (Ab101), and GST-c-Jun(1–79) fusion protein have been described previously (12, 21, 22). Fluorogenic substrates of caspase-1 (acetyl-Tyr-Val-Ala-Asp-4-methylcoumaryl-7-amide) and caspase-3 (acetyl-Asp-Glu-Val-Ala-Asp-4-methylcoumaryl-7-amide) were obtained from Peptides International (Louisville, KY). Fluorogenic substrate of caspase-8 (acetyl-Ile-Glu-Asp-Val-Ala-Asp-4-methylcoumaryl-7-amide) from Upstate Biotechnology. Coumarin was purchased from Sigma. TNF- $\alpha$  and caspase inhibitor

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were purchased from CalBioChem (La Jolla, CA). CHX was purchased from Sigma Chemical Co. (St. Louis, MO).

**Preparation of Soluble TRAIL.** A PCR fragment encoding soluble FLAG-TRAIL (amino acids 95–281) was cloned into the His-tag vector pET15b (12). The construct was then transformed into *Escherichia coli* BL21 (DE3) competent cells (Novagen, Madison, WI). His-FLAG-TRAIL recombinant protein was purified from bacterial lysates by using nickel chelate affinity chromatography. The concentration of purified protein was determined by the Bradford method (Bio-Rad, Hercules, CA) using BSA as standard. The purity of samples was assessed by electrophoresis.

**Analysis of Apoptosis.** After treatments, floating cells were collected by centrifugation at 1000 × g for 5 min, and attached cells were first trypsinized and then harvested by centrifugation. Apoptotic cells were assayed by using annexin-V-FITC staining kit (PharMingen, San Diego, CA). Briefly, the harvested cells were washed twice with ice-cold PBS and resuspended in a binding buffer containing both annexin-V-FITC and propidium iodide. After incubation at room temperature for 15 min, samples were analyzed by FACS Scan. Apoptosis was presented as the percentage of cells stained positive for annexin-V-FITC but negative for propidium iodide.

**Reverse Transcription-PCR Analysis of TRAIL Receptor Expression.** Total RNA was extracted with TRIzol reagent. cDNA was synthesized by using the SuperScript Preamplification System (Life Technologies, Inc., Gaithersburg, MD) and treated with 0.1 unit/μl *E. coli* RNase H at 37°C for 20 min. PCR was performed in a 50-μl reaction mixture containing cDNA (derived from 50 ng of total RNA), 1 unit of Taq DNA polymerase, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 μM deoxynucleotide triphosphate (each), and 200 ng/μl of each primer. Amplification was run for 35 cycles, with each cycle of PCR consisting of 1 min of denaturation at 94°C, 1 min of annealing at 60°C, and 2 min of extension at 72°C. PCR products were analyzed by agarose gel (1.5%) electrophoresis and photographed under UV light. Nucleotide sequences of PCR primers (23) used were as follows: DR4, 5'-CTG AGC AAC GCA GAC TCG CTG TCC AC-3' (sense), 5'-TCA AAG GAC ACG GCA GAG CCT GTG CCA T-3' (antisense); DR5, 5'-ATG GAA CAA CGG GGA CAG AAC-3' (sense), 5'-TTA GGA CAT GGC GTC TGC ATT AC-3' (antisense); DcR1, 5'-ACC CTA AAG TTC GTC GTC GTC ATC-3' (sense), 5'-TCA AAC AAA CAC AAT CAG AAG CAC-3' (antisense); and DcR2, 5'-CTT TTC CGG CGG CGT TCA TGT CCT TC-3' (sense), 5'-GTT TCT TCC AGG CTG CTT CCC TTT GTA G-3' (antisense). RT-PCR analysis of β<sub>2</sub>-microglobulin was described previously (24).

**Caspase Activity Assay.** After TRAIL treatment, cells were washed twice with ice-cold phosphate-buffered saline and lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 50 mM β-glycerophosphate, 15 mM MgCl<sub>2</sub>, 15 mM EDTA, 100 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and 150 mg/ml digitonin. After being left on ice for 30 min, cell lysates were passed through a 23-gauge needle three times and cleared by centrifugation. Caspase activity was determined by fluorogenic assays described previously (25).

**Immunocomplex Kinase Assay of JNK1 Activity.** After TRAIL stimulation, cells (in a 60-mm plate) were washed twice with ice-cold phosphate-buffered saline and lysed in a 250-μl buffer containing 10 mM Tris-HCl (pH 7.1), 50 mM NaCl, 50 mM NaF, 100 μM Na<sub>3</sub>VO<sub>4</sub>, 30 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 5 μM ZnCl<sub>2</sub>, 2 mM iodoacetic acid, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100. Cell lysates were homogenized by passing through a 23-gauge needle three times before incubation on ice for 30 min. Homogenates were cleared by centrifugation at 12,500 × g for 20 min and were assayed for JNK1 activity by using immunocomplex kinase assays described previously (22).

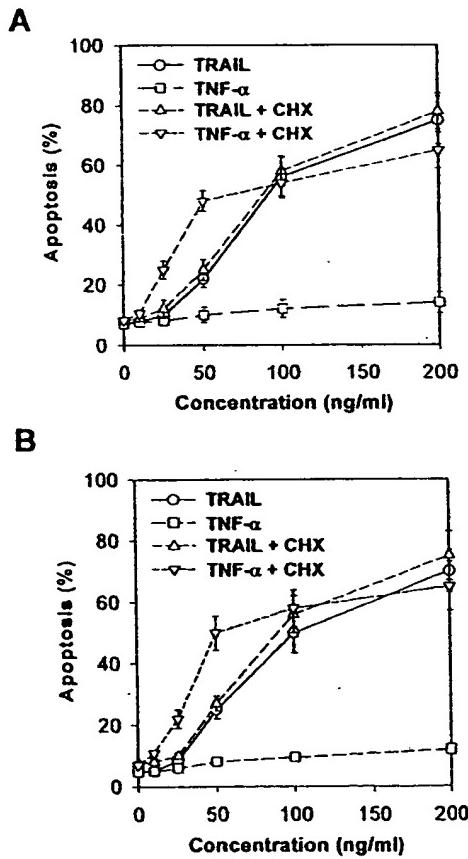
**Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay.** Nuclear extracts of PC-3 (10<sup>6</sup> cells) were prepared as described previously (26). For each binding reaction, 10 μg of nuclear protein, as determined by the Bradford method (Bio-Rad), were incubated with 1 ng of labeled, double-stranded NFκB oligonucleotide (~70,000 cpm) in a 10-μl binding buffer containing 0.5 μg poly(deoxyinosinic-deoxycytidylic acid), 9% glycerol, 10 mM HEPES (pH 7.9), 60 mM KCl, 1 mM EDTA, and 1 mM DTT at room temperature for 30 min. The resulting DNA-protein complexes were resolved in a 5% nondenaturing polyacrylamide gel at 5 W in Tris/glycine buffer (pH 8.4). The gel was dried and autoradiographed. NFκB binding oligonucleotide (sense, 5'-AGT TGA GGG GAC TTT CCC AGG C-3'; antisense, 5'-GCC TGG GAA AGT CCC CTC AAC T-3'; Promega Corp., Madison, WI) was labeled at the 5' end with [ $\gamma$ -<sup>32</sup>P]ATP using T4 kinase (Life Technologies, Inc.). The labeled oligonucleotides were purified by passing

through a G-25 column. Competition analysis was performed by adding a 25-fold molar excess amount of unlabeled NFκB oligonucleotides.

## RESULTS

**TRAIL, but not TNF-α, Induces Apoptosis in PC-3 and DU145 Cells.** Both PC-3 and DU145 are androgen-independent cell lines and are widely used in the study of advanced prostate cancer. Accordingly, we examined the sensitivity of PC-3 and DU145 cells to TRAIL-induced apoptosis. Treatment of PC-3 cells with TRAIL caused a dose-dependent apoptotic cell death, as measured by annexin-V-FITC staining (Fig. 1A). The induction of apoptosis became evident after 6 h of treatment with 50 ng/ml of TRAIL and reached 70% when TRAIL concentration increased to 200 ng/ml. In contrast, little cell death was observed in the cells treated with increasing concentrations of TNF-α for 36 h (Fig. 1A). However, PC-3 cells became sensitive to TNF-α killing in the presence of CHX (2 μM). Interestingly, pretreatment with CHX had little effect on TRAIL-induced apoptosis. Similar results were obtained when DU145 cells were treated with TRAIL and TNF-α in the presence or absence of CHX (Fig. 1B).

**Induction of Apoptosis by TRAIL Depends on Caspases.** Activation of caspases is a crucial event in apoptosis induced by various apoptotic agents (27). To provide a mechanistic insight into the



**Fig. 1.** Induction of apoptosis by TRAIL and TNF-α in androgen-independent prostate cancer cells. PC-3 (A) or DU145 (B) cells were treated with the indicated concentrations of TRAIL for 6 h or with TNF-α for 36 h in the absence or presence of CHX (2 μM). Apoptotic cells were analyzed by FACS as described in "Materials and Methods." The percentage of apoptosis represents the number of cells stained positive for annexin V of the total number of cells tested. The data points are means for three independent experiments; bars, SE.

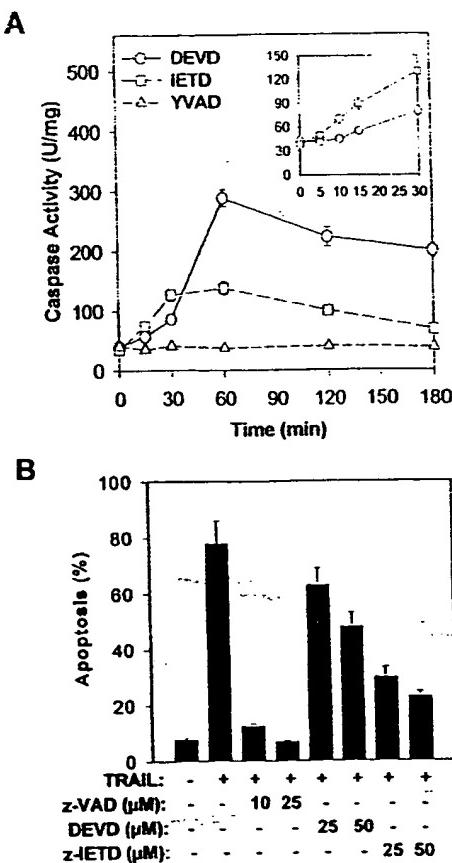


Fig. 2. The roles of caspases in TRAIL-induced apoptosis. *A*, time course of caspase activation. PC-3 cells were treated with TRAIL (100 ng/ml) for different time periods. The cells were then harvested and assayed for caspase activity by incubation of 10 μg of total cytosolic protein with 200 μM fluorogenic substrates Ac-DEVD-CMA, Ac-YVAD-CMA, or acetyl-Ile-Glu-Thr-Asp-methylcoumarin-7-anide in a 50-μl assay buffer. The release of amino-4-methyl-coumarin was monitored with a fluorescence spectrophotometer. Enzyme activity is expressed as units (pmol/min) per mg protein. *B*, inhibition of TRAIL-induced apoptosis by caspase inhibitors. PC-3 cells were treated with the indicated concentrations of caspase inhibitors z-VAD-fmk, Ac-DEVD-CHO, or z-IETD-fmk for 1 h prior to stimulation with TRAIL (100 ng/ml) for 6 h. The percentage of apoptotic cells was determined by annexin-V-FITC staining. Data presented are means of three independent experiments; bars, SD.

TRAIL-induced apoptosis, we monitored the activities of several caspases in PC-3 cells. As shown in Fig. 2*A*, TRAIL (100 ng/ml) stimulated DEVD- and IETD-specific activities in a time-dependent manner. In contrast, little YVAD-specific activity was observed over the same time period. Further time course study (as shown inside Fig. 2*A*) revealed that the induction of IETD-specific activity was early and preceded the induction of DEVD-specific activity, consistent with the notion that caspase-8 activity is upstream of caspase-3-like proteases in DR-mediated apoptosis.

To demonstrate an important role of caspases in TRAIL-induced apoptosis, we examined the effects of caspase inhibitors. As shown in Fig. 2*B*, z-VAD-fmk, a general inhibitor of caspases, blocked TRAIL-induced cell death in a dose-dependent manner. At a concentration of 25 μM, z-VAD-fmk completely abolished apoptosis. Pretreatment with z-IETD-fmk, a specific inhibitor of caspase-8, and Ac-DEVD-CHO, a specific inhibitor of caspase-3-like proteases, also significantly inhibited TRAIL-induced apoptosis in a dose-dependent fashion. Thus, TRAIL induces apoptosis in PC-3 cells through a caspase-dependent mechanism.

#### TRAIL-induced Apoptosis in PC-3 Cells Is Mediated by DR4.

TRAIL has been shown to be able to induce cell death through DR4, DR5, or both, depending on the expression of these DRs in the target cells. To identify TRAIL receptors that are responsible for the induction of apoptosis in PC-3 cells, we conducted reverse transcription-PCR analysis. A strong cDNA band derived from DR4 mRNA was observed, whereas DR5, DcR1, and DcR2 mRNA transcripts were undetectable (Fig. 3*A*). To exclude the possibility that the failure of detection of DR5, DcR1, and DcR2 mRNAs was attributable to the use of improper primers or unfavorable PCR conditions, we repeated the experiment with different primers or under different conditions. Again, no band was observed. Thus, the PC-3 cell line seems to selectively express DR4.

To provide further evidence for the role of DR4 in mediating TRAIL-induced apoptosis, we examined the effect of overexpression of DR4 on cell death. PC-3 cells were transfected with the increasing amount of plasmids encoding DR4 using a calcium-phosphate precipi-

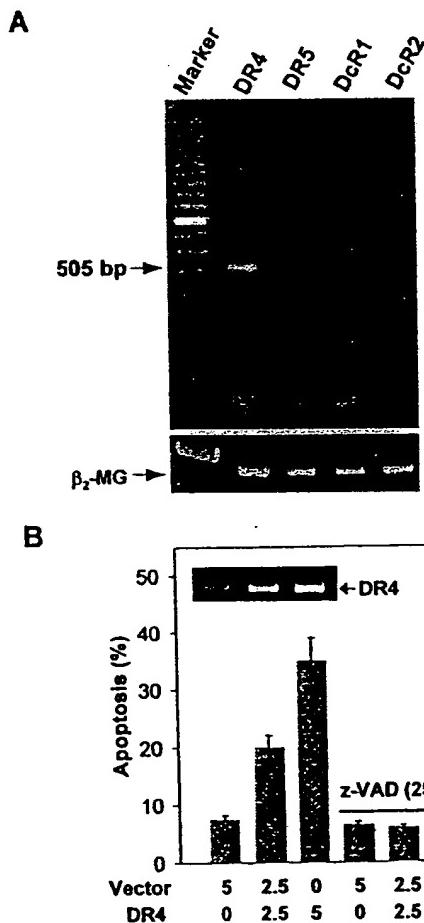


Fig. 3. Detection of DRs responsible for TRAIL-induced apoptosis in PC-3 cells. *A*, RT-PCR analysis of TRAIL receptor expression. Total RNA was isolated from PC-3 cells using the TRIzol reagent. Reverse transcription and PCR were performed as described in "Materials and Methods." PCR products were analyzed by agarose gel electrophoresis. Arrow, size of amplified sequence. Reverse transcription-PCR for human β<sub>2</sub>-microglobulin (β<sub>2</sub>-MG) mRNA was included as internal control. *B*, induction of apoptosis by DR4 overexpression. PC-3 cells were plated in six-well plates at a density of  $1.5 \times 10^5$  cells/well and were transfected with increasing amounts (μg) of DR4 expression vector in the presence or absence of z-VAD-fmk by the calcium-phosphate method. Total DNA in each well was adjusted to 5 μg with empty vector. Forty-eight h after transfection, expression of DR4 was analyzed by reverse transcription-PCR. Apoptotic cells were determined by annexin-V-FITC staining. The data presented are examples or means of three independent experiments; bars, SD.

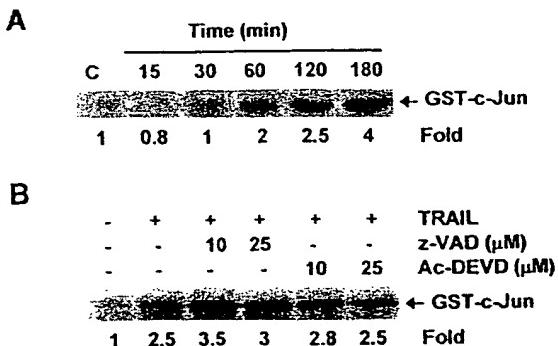


Fig. 4. Activation of JNK1 during the TRAIL-induced apoptosis. *A*, time course of JNK1 activation by TRAIL. PC-3 cells were treated with 100 ng/ml of TRAIL for different time periods. Endogenous JNK1 activity was determined by an immunocomplex kinase assay using GST-c-Jun(1–79) as substrate. *B*, effect of caspase inhibitors on JNK1 activation by TRAIL. PC-3 cells were treated with the indicated concentrations of z-VAD-fmk or Ac-DEVD-CHO for 1 h and then were stimulated with TRAIL (100 ng/ml) for 2 h. The cells were harvested and assayed for JNK1 activity as described in *A*. Data shown are examples of three separate experiments.

itation method (28). To eliminate transfection-related toxicity, the total amount of DNA in each transfection was adjusted to 5  $\mu$ g with empty vector. Forty-eight h after transfection, the expression of DR4 was analyzed by RT-PCR, and the apoptotic cell death was analyzed by annexin-V-FITC staining described above. As shown in Fig. 3*B*, when overexpressed, DR4 induced apoptosis in a dose-dependent manner. The addition of z-VAD-fmk (25  $\mu$ M) completely blocked this induction. Thus, overexpressed DR4 was able to mimic TRAIL to induce caspase-dependent apoptosis.

**TRAIL Stimulates JNK1 Activity, Which Appears to Be Independent of Caspases and Apoptosis.** In addition to the activation of caspases, cross-linking of DRs by TNF- $\alpha$  or FasL also has been shown to initiate kinase cascades, leading to the activation of JNK (29). To examine whether TRAIL is able to activate this pathway, we treated PC-3 cells with TRAIL for different time periods. As determined by immunocomplex kinase assays, TRAIL stimulated JNK1 activity, which occurred 1 h after treatment and reached a maximum at 3 h (Fig. 4*A*). Interestingly, the activation of JNK1 by TRAIL appears to be modest, with a maximum of 4-fold induction over control being observed.

Because TRAIL-induced JNK1 activity was relatively delayed compared with the induction of caspase activity (Figs. 2*A* and 4*A*), we next examined whether JNK1 activation by TRAIL depends on caspases. PC-3 cells were treated with the different concentrations of caspase inhibitor, z-VAD-fmk or Ac-DEVD-CHO, prior to the challenge with TRAIL. Neither z-VAD-fmk nor Ac-DEVD-CHO inhibited JNK1 activation by TRAIL (Fig. 4*B*). Instead, pretreatment with z-VAD-fmk slightly enhanced TRAIL-induced JNK1 activity. These data suggest that activation of JNK1 by TRAIL is independent of caspases.

**A Dominant-Negative Mutant of JNK1 Inhibits JNK1 Activation by TRAIL But Does Not Affect Apoptosis.** Given the fact that TRAIL-induced apoptosis is completely blocked in the presence of 25  $\mu$ M z-VAD-fmk whereas TRAIL-induced JNK1 activity is intact, we speculate that JNK1 activation may not be essential for the induction of apoptosis by TRAIL. To test this hypothesis, we used JNK1(APF), a dominant-negative mutant of JNK1, which has been shown previously to inhibit apoptosis induced by  $\gamma$ -radiation (21) and UVB (30). As shown in Fig. 5*A*, overexpression of JNK1(APF) inhibited TRAIL-induced JNK1 activity in a dose-dependent fashion. However, inhibition of JNK1 activation by JNK1(APF) had little effect on TRAIL-induced apoptosis, as determined by annexin-V-FITC staining (Fig. 5*B*).

**TRAIL Weakly Stimulates NF $\kappa$ B Activity in PC-3 Cells.** Another signaling pathway that is activated upon the cross-linking of DRs by TNF and FasL is NF $\kappa$ B (19). Accordingly, we asked whether TRAIL is able to activate the NF $\kappa$ B pathway. As measured by electrophoretic mobility shift assay, TRAIL induced a detectable NF $\kappa$ B binding activity at 1 and 3 h (Fig. 6*A*). However, under similar conditions, TNF- $\alpha$  induced a huge shift band. Thus, compared with TNF- $\alpha$ , TRAIL stimulated a weaker NF $\kappa$ B activity in PC-3 cells.

Previous studies have suggested that NF $\kappa$ B pathway may prevent cell death induced by death ligands and cancer chemotherapy drugs (31, 32). Strong activation of NF $\kappa$ B by TNF- $\alpha$  may therefore provide a reasonable explanation for the resistance of these prostate cancer cells to TNF- $\alpha$ . To test whether TNF- $\alpha$ -induced NF $\kappa$ B activation confers cross-resistance to TRAIL-induced apoptosis, we pretreated PC-3 cells with TNF- $\alpha$  prior to stimulation with TRAIL. Regardless of the duration of TNF- $\alpha$  treatment, no significant effect was observed on the induction of apoptosis by TRAIL (Fig. 6*B*), suggesting that distinct signaling components were involved in cell death induced by TRAIL and TNF- $\alpha$ .

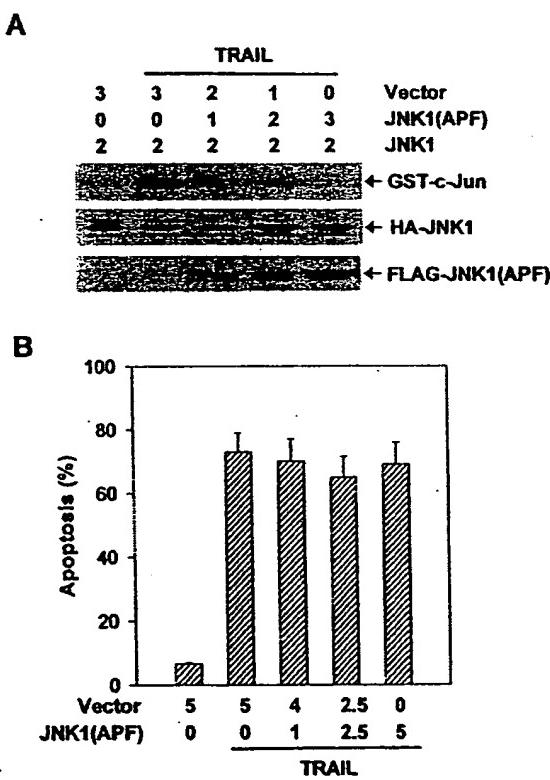
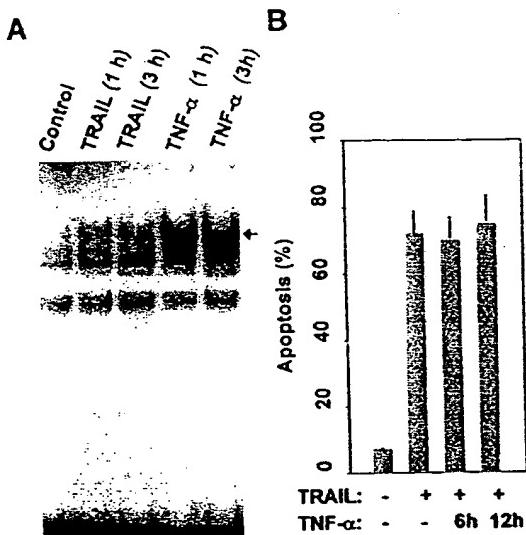


Fig. 5. Roles of JNK1 in TRAIL-induced apoptosis. *A*, effect of a dominant-negative JNK1 on TRAIL-induced JNK1 activation. PC-3 cells were plated in six-well plates and transfected with 2  $\mu$ g of plasmid encoding HA-JNK1 and different amounts ( $\mu$ g) of the empty vector or the vector encoding FLAG-JNK1(APF), a dominant-negative mutant of JNK1, by the calcium-phosphate method. After transfection, cells were treated with TRAIL (100 ng/ml) for 3 h. Exogenous JNK1 was immunoprecipitated with monoclonal anti-HA antibody (12CA5; Boehringer Mannheim, Indianapolis, IN) and assayed for kinase activity using GST-c-Jun as substrate. The expression levels of HA-JNK1 or FLAG-JNK1(APF) were determined by Western blotting with the specific antibody against HA or FLAG (M2; Sigma, respectively). *B*, effect of JNK1(APF) on TRAIL-induced apoptosis. PC-3 cells were transfected with increasing amounts ( $\mu$ g) of JNK1(APF) expression vector. The total amount of DNA in each well was adjusted to 5  $\mu$ g with empty vector. Forty-eight h after transfection, cells were stimulated with 100 ng/ml of TRAIL for 6 h. Apoptotic cells were analyzed by annexin-V-FITC staining. Columns, means for three individual experiments; bars, SE.



**Fig. 6.** The roles of NF-κB in TRAIL-induced apoptosis. *A*, effect of TRAIL on NF-κB activity. Nuclear extracts were prepared from PC-3 cells treated with TRAIL (100 ng/ml) or TNF-α (50 ng/ml) from 1 to 3 h. NF-κB binding activity was measured by electrophoretic mobility shift assays described in "Materials and Methods." Arrow, NF-κB/DNA complex. *B*, effect of NF-κB activation on TRAIL-induced apoptosis. PC-3 cells were pretreated with TNF-α (50 ng/ml) for 6 or 12 h prior to challenge with TRAIL (100 ng/ml) for 6 h. Apoptotic cells were determined by annexin-V-FITC staining. Columns, means for three individual experiments; bars, SE.

## DISCUSSION

Prostate cancer cells, especially the androgen-independent types, are resistant to most routinely used chemotherapeutic drugs (5). In the present study, we show that TRAIL is able to induce massive apoptosis in androgen-independent PC-3 and DU145 prostate cancer cells. The induction of apoptosis by TRAIL depends on the activation of caspases and is mediated by the death receptor DR4.

Although we have observed a strong activation of DEVDases or caspase-3-like proteases, we were unable to detect any significant changes in the activity of YVADases or caspase-1-like proteases, the caspases that have been shown to directly activate caspase-3 *in vitro* (33). This raises the possibility that caspases other than caspase-1-like proteases are involved in the activation of caspase-3-like proteases. Previous studies with FasL- or TNF-induced apoptosis indicate that cross-linking of Fas or TNF receptors results in the recruitment of death domain-containing proteins (FADD/MORT1) that, in turn, interact with caspases such as caspase-8, thereby initiating a caspase cascade (19). Indeed, treatment of PC-3 cells with TRAIL induced caspase-8 activity, and z-IETD-fmk, an inhibitor of caspase-8, attenuated TRAIL-induced apoptosis, implicating the important role of this caspase. Whether the activation of caspase-8 by TRAIL involves a FADD-dependent mechanism is currently not clear. A recent report shows that cells from FADD-deficient mice are resistant to apoptosis induced by Fas, TNFR1, and DR3 but show full responsiveness to DR4, which supports the existence of a FADD-independent pathway that couples TRAIL to caspases (34). However, transfection studies with the dominant-negative FADD have yielded conflicting results (12, 13). Thus, elucidation of adapter protein(s) for TRAIL receptors warrants further studies.

JNK, also called stress-activated protein kinase, is a member of the mitogen-activated protein kinase family (35). A number of studies have identified JNK to be involved in the regulation of apoptosis. Activation of JNK has been shown to be required for the induction of apoptosis by stress stimuli such as growth factor withdrawal (36),

γ-radiation, and UVC (21). However, the role of JNK pathway in receptor-induced apoptosis seems to vary depending on the nature of DRs and the cell lines. For example, in Jurkat cells, anti-Fas (CH-11) strongly induces caspase-dependent activation of JNK1, which seems to be dispensable for Fas-mediated apoptosis (21, 37), whereas in 293 and HeLa cells, JNK activation by FasL is essential for the induction of apoptosis (38). With respect to TNF signaling, JNK activation has been dissociated with the FADD-mediated apoptotic pathway (39). In this study, we demonstrated that TRAIL was capable of activating the JNK signaling pathway, which appeared to be independent of caspases, as indicated by the delayed activation pattern and the lack of effects of caspase inhibitors. However, direct interference with JNK1 activity by overexpression of a dominant-negative mutant of JNK1 did not affect the induction of apoptosis by TRAIL. Consistent with this observation, a recent study also indicates that, although TRAIL can activate JNK through both caspase-dependent and caspase-independent pathways, activation of JNK may not be sufficient for the induction of apoptosis (40). Therefore, we conclude that activation of JNK pathway may not be directly involved in the induction of apoptosis by TRAIL.

NF-κB is a transcription factor that plays an important role in immunological and inflammatory responses by inducing a number of proinflammatory cytokine genes. Recent reports suggest that NF-κB is also involved in the regulation of apoptosis (31). Activation of NF-κB has been demonstrated to suppress the induction of apoptosis by a variety of apoptotic agents, including certain routinely used anticancer drugs (32), and thereby may counteract their therapeutic efficacy. NF-κB activation is also believed to contribute to the severe inflammatory response syndrome after systemic administration of certain TNF-α doses. We observed that, in PC-3 cells, TNF-α strongly induced NF-κB activity but failed to induce apoptosis unless in the presence of protein synthesis inhibitor, supporting the view that activation of NF-κB may induce antiapoptotic genes and protect cells against TNF-α-induced apoptosis. However, unlike TNF-α, TRAIL only weakly induces NF-κB activity, as shown in this study as well as by others (12, 16). Most interestingly, activation of NF-κB by pretreatment with TNF-α has little effect on the induction of apoptosis by TRAIL (Fig. 6B), suggesting that distinct pathways may exist in regulating TRAIL-induced apoptosis. Alternatively, the differential effects of NF-κB on TRAIL- and TNF-α-induced apoptosis may reflect the different natures of their receptors to recruit the antiapoptotic proteins, the expression of which is under the control of NF-κB because the NF-κB-stimulated inhibitory effect on TNF-α-induced apoptosis often occurs at the level of receptor complex (41). Future elucidation of TRAIL receptor-associated proteins will shed light on this issue.

In summary, this study demonstrates that TRAIL induces massive apoptotic cell death in androgen-independent prostate cancer cells. The induction of apoptosis is mediated by caspase cascades. Although TRAIL also activates JNK1 and, to a lesser extent, stimulates NF-κB activity, modulation of these pathways has no significant effect on TRAIL-induced apoptosis. Furthermore, the induction of apoptosis by TRAIL is independent of p53 because PC-3 cells do not express functional p53 (42). Thus, TRAIL may have therapeutic potential against advanced prostate cancer.

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